Ultraviolet Spectroscopy of β-Lactoglobulin Films

β-Lactoglobulin transferred from a film balance to the surface of quartz plates exhibits a molar (residue) absorptivity of 11,950 liters $mole^{-1}$ cm^{-1} at 193–194 nm compared to 9,200 liters $mole^{-1}$ cm^{-1} observed with isotropic solutions of the labeled protein and 9,600 liters $mole^{-1}$ cm^{-1} observed with solutions of unlabeled protein. It is proposed that the main factor responsible for the enhanced absorptivity is the effect of orienting a planar sample in the light beam of the spectrophotometer. Solvent effects and changes in the conformation of the protein are unable to account for the increased absorptivity observed in films.

INTRODUCTION

In the course of investigating lipid–protein interactions in monolayers, the conformation of the protein, β-lactoglobulin (β-lg), in the films was determined by circular dichroism (CD) spectroscopy (1). Ultraviolet (UV) spectroscopy was used to monitor the amount of film transferred from the air–water interface to the surface of quartz plates (1). The amount of β-lg in the transferred films was calculated from the UV spectra using the molar (residue) absorptivity determined on neutral solutions of the protein (1, 2). As was pointed out, this procedure ignores solvent and orientation effects (1), not to mention possible changes in conformation of the protein upon spreading into a film with subsequent transfer to a quartz surface.

The recent development of a nonperturbing derivatization of β-lg with a radiolabel (3) provides a means of independently assaying the protein in films. The combination of liquid-scintillation counting of labeled β-lg with UV and CD spectroscopy permits study of the conformation and orientation of the protein in the film. The results are presented below.

EXPERIMENTAL

Materials. β-Lactoglobulin genetic variant A was prepared by the method of Aschaffenburg and Drewry (4). Carbon-14 formaldehyde, Protosol, and Aquasol-2 were purchased from New England Nuclear[1] (Boston, Mass.). The film balance, subphases, and spreading solvents have been described (1), as have the quartz plates and plate holders for CD spectroscopy (5). Dialamatic microdispensers (Drummond Scientific Co., Broomall, Penn.) were used for monolayer spreading. For UV spectroscopy special cylindrical plate holders were constructed to allow mounting of the quartz plates in two sets, rotated 90° with respect to each other around the axis of the incident beam. One holder was for the transferred films and one for clean quartz plates used as a blank in the UV runs.

Methods. Reductive methylation of the lysine groups of β-lactoglobulin A was done by the method of Rowley *et al.* (3) using the moderate derivatization procedure. All operations involving radioactive material were performed in a fume hood with the operator wearing protective clothing and a face mask. Amino acid analysis of a control synthesis using "cold" formaldehyde showed that 4-5 lysines (of 15) were modified, in agreement with the published method (3). The concentration of protein in solution was determined from the UV absorption at 280 nm with $E^{1\%}_{1\,cm} = 9.6$ (6). Monolayers of β-lg were spread from phosphate buffer, pH 7, by the Trurnit rod technique (7), and transferred to quartz plates as previously described (1, 5). Six or eight plates were used for each monolayer run, with an identical number of clean plates in the reference beam. Transfer was effected at high film concentration by decreasing the film area at the same rate (area/unit of time) that the quartz plates were lifted through the air–water interface. Separate pressure–area runs with a Wilhelmy plate, but no quartz transfer plates suggested that the films were transferred at 25–30 mN/m. UV spectra of the transferred films were obtained on a Cary-14 spectrophotometer adapted to scan to 180 nm with the monochromometer and sample compartments purged with dry nitrogen. All plates from a given monolayer run were mounted together in orthogonal pairs at 0 and 90° from the perpendicular for recording of the UV spectrum. The plates were also scanned individually for uniform film deposition with a Gilford-252 spectrophotometer-gel scanner set at 203 nm. The quartz plates were assayed for protein by liquid scintillation analysis of the C-14 labeled protein. Each plate was treated twice by ultrasonic agitation at 50°C with Protosol tissue solubilizer and methanol rinse. The solubilized protein samples were neutralized with glacial acetic acid to eliminate chemiluminescence and mixed with Aquasol for assay with a Beckman LS-8100 scintillation counter. Sufficient counts were taken to insure a 1% or less standard deviation in

[1] Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

the final data. Background checks on the quartz plates showed that virtually all protein had been removed by the solubilizing procedure. Recovery of a known amount of labeled protein evaporated from solution onto a clean quartz plate was quantitative.

RESULTS AND DISCUSSION

Previously, UV spectroscopy was employed to determine the β-lg concentration in films used for CD spectroscopy (1) where the mean residue absorptivity of the protein in solution (1, 2) was used in the calculations. As was pointed out, this procedure ignored solvent and orientation effects and permitted only estimates of the CD transitions to be made (1). Ignoring the solvent effect turns out to be of little consequence for β-lg, as the results of Fig. 1 show, where only a small difference is seen between the spectra of films run in dry and aqueous-saturated nitrogen atmosphere. In this example, the spectrum was first recorded with the films bathed in dry nitrogen, then the sample and reference compartments were flushed with nitrogen at 99–100% RH for several hours, and a second spectrum was run. Most lyophillized proteins contain 0.05–0.07 g water per g protein (E. Berlin, private communication) and β-lg contains 0.78 g water/g protein at saturation (8) and 0.83 g water/g protein in crystals (9), hence the films used in this work probably contained between 0.05 and 0.8 g water/g protein at the extremes. The results of Fig. 1 show that the UV spectrum of a β-lg film is largely unaffected by moisture within these approximate limits. However, the molar absorptivity at 193 nm in the spectra of both the wet and "dry" films is more intense than that observed in the spectrum of an isotropic solution of β-lg, as shown by calculations from several spectra

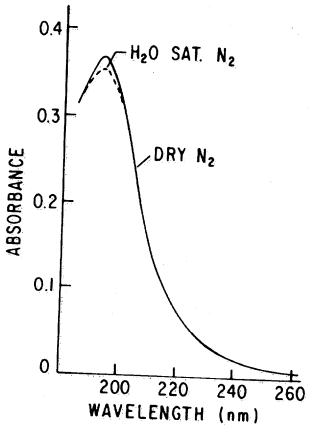

FIG. 1. Ultraviolet spectra of β-lactoglobulin films mounted on six quartz plates. Each spectrum was obtained with either dry or saturated nitrogen atmosphere in both the sample and reference compartment of the spectrophotometer as indicated.

where labeled proteins were employed. With liquid scintillation counting used to assay for protein in the films, calculations from nine UV runs gave a residue absorptivity of 11,950 liters $mole^{-1}$ cm^{-1} with 95% confidence limits of ±280 liters $mole^{-1}$ cm^{-1}. This is 25% higher than the absorptivity of 9600 liters $mole^{-1}$ cm^{-1} calculated from the results of McDiarmid obtained with aqueous solutions (2) and confirmed in the author's laboratory. A solution of derivatized protein gave an absorptivity of 9,200 liters $mole^{-1}$ cm^{-1}, hence labeling of the protein was not the cause of the hyperchromicity observed in films. For the film work, both manual and automated balances were used with consistent results. The data include results from one monolayer spread from acidic chloroform/methanol as previously described (1) which gave an absorptivity of 11,980 liters $mole^{-1}$ cm^{-1}, suggesting that the spreading solvent was not retained in the dried film.

Calculation of the absorptivity from the protein concentration and the UV spectra depends on an accurate knowledge of the surface area of the transferred film and assumes that the concentration is reasonably uniform over the area covered. Densitometer tracings of the coated plates shown in Fig. 2 indicate that these expectations are fulfilled. The sharp spikes are diffraction effects from the edges of the quartz plates. The raised plateau represents the protein-coated portion of the plate. The depressed portion of the tracings are areas where the Teflon tape covered the plate during film transfer. Comparing measurements on the Teflon-wrapped plates to the densitometer tracings showed that the protein coated the exposed surface of the quartz to within experimental error. The protein coating was reasonably smooth with no preferential buildup in the center of the plates which could account for increased UV absorption. Rotation of the protein coated plates in the UV spectrometer caused less than 2% change in the UV absorbance at 193 nm. The plate to plate film concentration was reasonably uniform within each run as judged by the average relative standard deviation of 10% in the individual plate counts. The enhanced absorption thus does not appear to be due to any artifacts of sample preparation.

Changes in conformation can cause the UV absorptivity of polypeptides and proteins to vary (10), but this is unlikely to be the cause of the increased absorption observed with β-lg films. In solution where the protein is in the native state, β-lg(A) exists mostly in the β-sheet and unordered conformations (11), both of which exhibit high absorptivities as compared to that observed with the α-helix (10). For example, suppose one adopts the estimate of Timasheff *et al.* of the structural composition of β-lg(A) as 13% α-helix, 40% β-sheet, and 47% unordered (11), and chooses as estimates of the UV absorptivity for the α-helix 4400 liters $mole^{-1}$ cm^{-1}, for β-sheet 7700 liters $mole^{-1}$ cm^{-1}, and for the unordered conformation 7100 liters $mole^{-1}$ cm^{-1} (10). One can show that converting β-lg to 100% β-structure will result in an absorptivity increase of only about 700 liters $mole^{-1}$ cm^{-1}. Furthermore, since

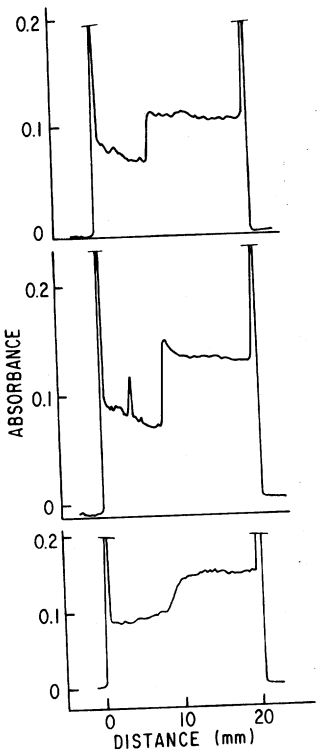

FIG. 2. Densitometer scans of individual protein-coated plates from different transfer runs. Wavelength was 203 nm. Note that not all absorbance scales are identical.

CD spectra indicate that α-helix is retained in β-lg films (1), and not converted to β-sheet during spreading, the above calculation probably overestimates the UV increase to be expected from conformation change. Timasheff *et al.* caution that the estimates of β-lg composition are to be regarded only as "working numbers" (11), hence the above calculation should not be taken too literally, but it does serve to show that most of the enhanced absorptivity observed in β-lg films must result from something other than a change of conformation in the protein.

The orientation of an absorbing species in the light beam of a spectrophotometer can definitely influence the absorption observed (12). In monolayer assemblies prepared as reported here, the planes of the films as mounted in the spectrophotometer are normal to the incident light beam. This means that the electric field vector of the incident light is coplanar with the film and with the absorption transition moments of the β-sheet portion of the film, which are also largely in plane (13). With such a favorable alignment of the electric field vector of light and the absorption transition moment of the polymer, as much as 50% enhancement in absorption can occur over that observed with isotropic solutions (14). The films of β-lg reported on here exhibited about 25% higher absorptivity than is observed with isotropic solutions. Since both CD (1) and IR spectroscopy (15) have shown that the β-lg monolayers compressed on a film balance contain a large amount of the β conformation, it seems reasonable to suggest that most of the enhancement in UV absorption observed here arises from the optical anisotropy of a planar sample containing a high proportion of β-sheet mounted normal to the incident light. The intensities of the CD transitions of the monolayer samples reported in Ref. (1) have probably been underestimated since the absorptivity of isotropic solutions of β-lg were used to calculate their concentrations in the films. The conclusions from that study however, remain intact.

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